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PIK3CA as an oncogene in cervical cancer

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Amplification of chromosome arm 3q is the most consistent aberration in cervical cancer, and is implicated in the progression of dysplastic uterine cervical cells into invasive cancer. The present study employed the 'positional candidate gene' strategy to determine the contribution of *PIK3CA*, which is located in 3q26.3, in cervical tumorigenesis. *PIK3CA* is known to be involved in the PI 3-kinase/AKT signaling pathway, which plays an important role in regulating cell growth and apoptosis. The results of comparative genomic hybridization show that the 3q26.3 amplification was the most consistent chromosomal aberration in primary tissues of cervical carcinoma, and a positive correlation between an increased copy number of *PIK3CA* (detected by competitive PCR) and 3q26.3 amplification was found in tumor tissues and in cervical cancer cell lines. In cervical cancer cell lines harboring amplified *PIK3CA*, the expression of gene product (p110α) of *PIK3CA* was increased, and was subsequently associated with high kinase activity. In addition, transformation phenotypes in these lines, including increased cell growth and decreased apoptosis, were found to be significantly affected by the treatment of specific PI 3-kinase inhibitor, suggesting that increased expression of *PIK3CA* in cervical cancer may result in promoting cell proliferation and reducing apoptosis. These evidences support that *PIK3CA* is an oncogene in cervical cancer and *PIK3CA* amplification may be linked to cervical tumorigenesis. *Oncogene* (2000) 19, 2739–2744.

Keywords: *PIK3CA*; p110α; cervical cancer; AKT; PI 3-kinase; 3q26.3

Introduction

Carcinoma of the uterine cervix is a common malignancy which affects women worldwide. Currently, it ranks as the number one cause of female cancer mortality, with more than 500 000 new cases each year among women in developing countries (Shah and Howley, 1996). Epidemiological studies have suggested that multiple risk factors are implicated in the tumorigenesis of cervical cancer, with human papillomavirus (HPV) considered the most dominant risk factor. HPV infection of the uterine cervix affects the immature metaplastic cells of the transformation zone, and results in the development of cervical intraepithelial neoplasia (CIN) of different severity. Although HPV infection is common in young sexually active females, only a small portion of females develop CIN, and even less develop invasive cancer (Shah and Howley, 1996). This pyramid shape of incidence distribution of HPV infection, CIN and cervical cancer, and the very slow progressive nature of CIN to carcinoma, suggest that events other than HPV infection are necessary for carcinogenesis. Among these events, genetic aberrations of growth-controlling genes, a universal feature of tumorigenesis, play a pivotal role.

Gene amplification is one of the essential mechanisms of oncogene activation. Thus, recurrent DNA copy number increases in tumors revealed by karyotypic abnormalities, such as double minute or homogeneously staining region, have been considered to be clues suggesting areas that may harbor putative oncogenes. On the basis of comparative genomic hybridization (CGH) studies, among chromosomal arms, gains and amplification in 3q have been consistently detected in many tumor types (for a review, Rooney *et al*., 1999). In search of the genes whose selection drives 3q amplification, a recent study has provided convincing evidences that *PIK3CA* at 3q26 (Volinia *et al*., 1994), which encodes the p110α catalytic subunit of phosphatidylinositol (PI) 3-kinase, is an oncogene in ovarian cancer (Shayesteh *et al*., 1999).

PI 3-kinase is a unique intracellular signal transducer characterized by its lipid substrate specificity (for review, Leevers *et al*., 1999; Marte and Downward, 1997). The binding of receptors associated with multiple growth factors, including PDGF and EGF, by the p85 subunit of PI 3-kinase results in activation of the catalytic subunit (p110α), which then catalyzes phosphorylation of PI 3,4-bisphosphate (PIP_2) into 3,4,5-triphosphate (PIP_3). One critical downstream target of this signaling pathway is the activation of protein-Ser/Thr kinase (AKT)-1, which subsequently effects multiple cellular processes, including increased cellular proliferation or decreased apoptosis, and contributes to tumorigenesis (Klippel *et al*., 1998; Kennedy *et al*., 1997; Tlsty, 1999). Because amplification in 3q is also the most consistent chromosomal aberration found in cervical cancer, and is implicated in the progression of dysplastic uterine cervical cells into invasive cancer (for review, Rooney *et al*., 1999; Ried *et al*., 1999), the present study sought to determine the contribution of *PIK3CA* in cervical tumorigenesis.

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Results and discussi n

Genomic imbalances were detected by CGH in 14 of 18 cervical carcinomas, and, consistent with previous studies of cervical cancer (for review, Rooney *et al.*, 1999; Ried *et al.*, 1999), 3q was the most common arm displaying chromosomal gain. Among 10 tumors showing a chromosomal gain at 3q, 3q26.3 was found in eight tumors, and was the most common site of copy number increases (Table 1 and Figure 1). We next explored whether *PIK3CA* was the target gene driving 3q26.3 amplification. The relative copy number of *PIK3CA* and the presence of *PIK3CA* amplification were detected by competitive PCR (PCR MIMICS). In the CGH and PCR MIMICS, the positive control cell line (Ovca) displayed 3q26.3 amplification and 3.9 copies of *PIK3CA*, and the negative control cell line (HBL-100) did not show such amplification and had no increase (2.1 copies) of *PIK3CA*. In 16 tumors simultaneously assayed by CGH and by PCR MIMICS, a positive correlation between 3q26.3 amplification and increased relative copy number of *PIK3CA* was found ($P=0.09$ by regression analysis) (Table 1). All tumors displaying 3q26.3 amplification detected by CGH were found to harbor $\geqslant 3.5$ copies of *PIK3CA*. However, CGH failed to detect a 3q26.3 amplification in five cases exhibiting $\geqslant 3.5$ copies of *PIK3CA*. The failure of CGH to detect increased *PIK3CA* in these five cases of cervical carcinoma might have resulted either from the relatively low resolution of CGH to detect small amplification fragments or from the confounding effects due to normal tissue contamination in primary tumor tissues when CGH was assayed.

In addition to *PIK3CA*, another possible gene within the 3q amplicon is *hTR*, which encodes the RNA component of human telomerase (Feng *et al.*, 1995). Telomerase plays an essential role in stabilizing telomere length and, consequently, contributes to the processes of cellular immortality and tumorigenesis. During cervical cancer development, differential expression of telomerase activity has been reported (Pao *et al.*, 1997). To distinguish between these two putative

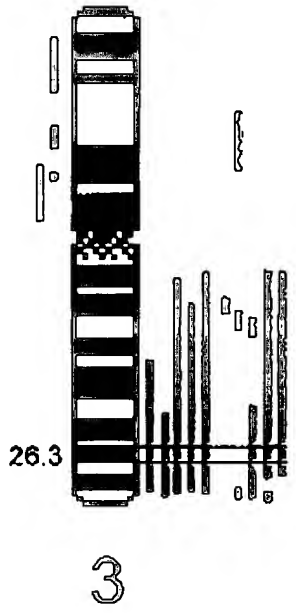

Figure 1 CGH imbalances of chromosome 3 detected in 18 cervical carcinomas. Ten tumors showed a chromosomal gain at 3q

Table 1 Analysis of 3q amplification, relative copy number of PIK3CA and HPV infection status in primary cervical carcinoma tissues and cervical cancer cell lines[a]

Case no/cell line	*3q amplification*	*3q26.3 amplification*	*Relative copy no. of* PIK3CA	*HPV infection (type)*
Cervical carcinoma				
57C	+	+	5.5	+ (16)
29C	+	+	4.7	–
44C	+	+	4.5	+ (52)
46C	+	+	4.0	+ (16)
56C	+	+	3.6	+ (16)
38C	+	+	3.5	+ (16)
25C	+	–	6.0	+ (16)
80C	+	–	3.5	+ (16)
30C	–	–	3.7	+ (16)
71C	–	–	3.7	+ (58)
35C	–	–	3.6	+ (16)
21C	–	–	3.1	+ (16)
68C	–	–	3.0	+ (16)
53C	–	–	2.8	–
67C	–	–	2.8	+ (16)
48C	–	–	1.9	+ (16)
15C	+	–	ND[b]	+ (33)
17C	+	–	ND	+ (16)
Cell line				
C-33A	+	+	3.7	–
ME-180	+	+	3.5	+ (18)
SiHa	+	–	2.5	+ (16)

[a]Genomic amplification in 3q or 3q26.3 was detected by CGH, and relative copy number of *PIK3CA* was measured by competitive PCR (PCR MIMICS). The presence of HPV infection and the type of HPV was identified from amplification of a fragment of the HPV L1 region by consensus primer PCR and subsequent restriction fragment length polymorphism analysis (Ku *et al.*, 1997). [b]Not done due to insufficient DNA

genes in 3q, we measured copy number changes of *PI3KCA* and *hTR* in 55 cervical cancer specimens using competitive PCR (PCR MIMICS). Whereas only 5% (3/55) of tumors expressed an increased copy number of *hTR*, 76%(42/55) revealed more than 2.5 copies of *PI3KCA* (among which 22 cases had more than 3.5 copies of *PIK3CA*). In addition, all the tumors that demonstrated *hTR* amplification also demonstrated *PIK3CA* amplification. These findings suggest that *PI3KCA* is the major candidate target of gene amplification at 3q26.3, and may contribute to cervical tumorigenesis.

To further explore the clues provided by primary tumor tissues, we examined the role of *PIK3CA* in cervical cancer cell lines by sequential experiments. As compared to the positive (Ovca) and negative (HBL-100) controls, all of the three cervical cancer cell lines tested show more than 2.5 copies of *PIK3CA*, suggesting a role of this gene during cervical tumorigenesis (Table 1). Among these lines, two were detected by CGH to display the 3q26.3 amplification. The failure to detect 3q26.3 amplification in SiHa, which was found to harbor an increased copy number of *PIK3CA* by PCR MIMICS, might be similar to that observed in primary tumor tissues, and was due to the low resolution of CGH to detect small amplification fragment. We next examined whether increased *PIK3CA* copy number contributed to elevated p110α expression. Given an equal amount of protein used in Western blotting (indicated by similar intensity of the band of internal control, Cdc2), compared to the Ovca cell line known to harbor increased *PIK3CA* and to display elevated levels of p110α (Shayesteh *et al*., 1999). Three cervical cancer cell lines expressed comparable (i.e. C-33A) or elevated (SiHa and ME-180) levels of p110α (Figure 2a). In contrast, it was expected that no or minor levels of p110α were detected in the negative control (HBL-100) and the normal fibroblast cell line (HS68) (Figure 2a).

In agreement with protein expression level, compared to Ovca, an increased activity of PI 3-kinase was detected in the three cervical cell lines, which harbored an increased copy number of *PIK3CA* and showed elevated expression of p110α (Figure 2b,c). In contrast, the normal cell line (HS68) and the negative control (HBL-100) demonstrated only minor p110α activity. These data indicate that elevated expression of p110α resulting from *PIK3CA* amplification in cervical cancer cells leads to increased PI 3-kinase activity.

The PI 3-kinase complex is composed of a catalytic subunit, p110α encoded by *PIK3CA*, and a regulatory subunit, p85. In ovarian cancer, increased PI 3-kinase activity resulting from increased p110α-p85 heterodimer formation is caused solely by the over-expression of p110α, and p85 does not seem to play a role in such increase, since all of the p110α is associated with p85, whereas most of the p85 is not associated with p110α (Shayesteh *et al*., 1999). In the present study, we also examined the contribution of p85, and also found p85 showed no increase in protein level. However, in cervical cancer, the possibility that p85 contributes to increased PI 3-kinase activity via particular epigenetic pathways cannot be excluded. The PI 3-kinase pathway can be activated by interleukin-4 (IL-4) stimulation, partially via affecting the protein tyrosine phosphorylation status of p85 (Imani *et al*., 1997). In high-grade

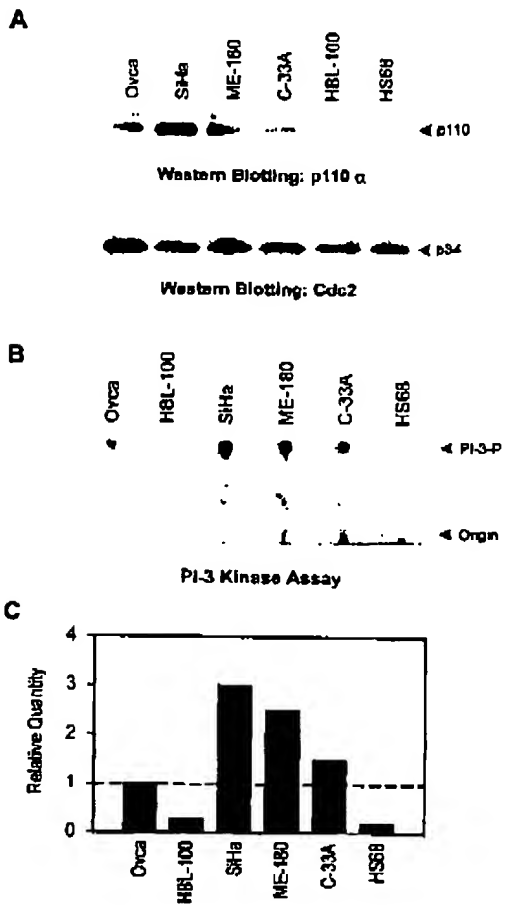

Figure 2 (a) Western blot analysis of p110α (encoded by *PIK3CA*) expression in cervical cell lines (SiHa, ME-180, and C-33A), ovarian cancer cell line (Ovca), human breast cell line (HBL-100), and normal human skin cell line (HS68). The Ovca cell line was known to harbor amplified *PIK3CA*, serving as a positive control, whereas the HBL-100 was known to be negative of *PIK3CA* amplification. All cervical cancer cell lines displayed an increase copy number (≥2.5 copies) of *PIK3CA*. Cdc2 served as an internal control to show equal amount protein used in individual cell lines in this assay. (b) PI 3-kinase activity in cell lines with different statuses of *PIK3CA* amplification and p110α expression. The ability to phosphorylate PI *in vitro* was measured for p110α protein immuno-precipitated from cellular lysates. (c) Relative levels (densitometric analyses) of PI -3 kinase activity in cell lines shown in b. The relative quantity of individual lines was indicated by the relative amount as compared to the activity of the positive control (Ovca)

CIN, IL-4 production in response to mitogen stimulation was significantly elevated in a subset of patients (Clerici *et al*., 1997), which may in turn activate p85 leading to increased PI 3-kinase activity. Furthermore, a recent study shows that, after the treatment with specific growth factors in cervical carcinoma cells, the p85 subunit can be phosphorylated, subsequently, resulting in activation of the PI 3-kinase pathway (Ebert *et al*., 1999).

We finally examined the biological effect of increased PI 3-kinase activity by treating cells with the PI 3-kinase inhibitor, LY294002. One test of PI 3-kinase function is to demonstrate the triggering of cell growth. Treatment with LY294002 resulted in a dose-dependent

decrease in cell number as indicated by MTT dye conversion in SiHa and ME-180 cells, implying that cell growth is attributable to increased PI 3-kinase activity. In contrast, the inhibition of cell growth was not obvious in the cell line HS68, without amplified *PIK3CA* and increased PI 3-kinase activity (Figure 3a). The other test of PI 3-kinase function is to demonstrate its effect in regulating apoptosis. Corresponding to cell growth assay, after treatment with LY294002, only the cells (SiHa and ME-180) which harbored increased *PIK3CA* copy number showed increase in levels of apoptotic DNA fragments (Figure 3b). In contrast, the normal cell line (HS68) showed no increase in apoptotic bands following LY294002 treatment. Thus, increased PI3-kinase activity associated with *PIK3CA* amplification appears to respond with apoptosis when kinase activity is inhibited. Increased PI3 kinase activity associated with PI3 kinase amplification contributes to tumorigenesis through increased cell proliferation or reduced apoptosis.

The present study employed the 'positional candidate gene' strategy to define *PIK3CA* as an oncogene contributing to cervical cancer formation. Taken together, the results of this study support the following three predictions: (i) the 3q26.3 amplified region, where *PIK3CA* is located, is the most frequent site of genomic amplification in cervical cancers, and an increased copy number of *PIK3CA* was found in a high proportion of tumor tissues having 3q amplification; (ii) expression of the gene product (p110α) of *PIK3CA* was increased, and was associated with high functional (PI 3-kinase) activity; (iii) *PIK3CA* amplification was associated with the biological effects of aberrant cell proliferation and apoptosis, both of which are directly linked to tumor formation. In addition, the possibility of the involvement of genetic amplification of *hTR*, the other candidate gene located at the same amplified region, has been shown to be minor. Further analysis of PI3-kinase activity in cell lines derived from the cervical epithelium that do not have increased *PIK3CA* will be helpful to strengthen our conclusion. However, the biological plausibility of a tumorigenic implication of *PIK3CA* amplification and the PI 3-kinase signal pathway has gained strong support from recent evidences showing that: (i) proliferative defect was demonstrated in mice homozygous for a deletion in *PIK3CA* (Bi *et al*., 1999); (ii) chicken cells could be transformed by the gene encoding the catalytic subunit of PI 3-kinase (Chang *et al*., 1997); (iii) inactivation of *PTEN*, a specific suppressor of the PI 3-kinase pathway, occurs frequently in multiple advanced cancers (for a review, Cantley and Neel, 1999). Accordingly, we suggest that increased PI 3-kinase activity resulting from *PIK3CA* amplification may be causally linked to the development of cervical cancer.

Among the various signaling pathways which are activated downstream of PI 3-kinase, the AKT pathway has attracted much attention because of its role in cell survival by triggering cell out-growth or inhibiting apoptosis (for review, Marte and Downward, 1997; Tisty, 1999; Datta *et al*., 1999). Though the present study did not examine the involvement of AKT pathway activation as a consequence of increased PI 3-kinase, the phenotypic changes observed in the *PIK3CA*-amplified cells treated with PI 3-kinase inhibitor, including decreased cell growth and increased apoptosis, along with current understanding of AKT pathway (for a review, Datta *et al*., 1999), provide clues in favor of the association between the AKT pathway and PI 3-kinase in cervical tumorigenesis. Furthermore, support for the involvement of AKT in activated PI 3-kinase pathway in cervical tumorigenesis comes from the demonstration that PI 3-kinase-dependent signaling pathway involving AKT phosphorylation can be induced in cervical carcinoma cell lines, and the phosphorylation of AKT can be blocked by the treatment of a specific inhibitor of PI 3-kinase (Ebert *et al*., 1999). The PI 3-kinase/AKT pathway regulates cell proliferation possibly by means of contributing to nuclear accumulation of cyclin D1 (Muise-Helmericks *et al*., 1998; Diehl *et al*., 1998), which consequently results in phosphorylation of retinoblastoma (Rb) protein and S phase entry in cell cycle. However, the consequence of increased *PIK3CA* in cervical cancer would be intriguing because Rb is usually inactivated by binding with HPV's E7 protein during cervical tumorigenesis. Thus, it is possible that the PI 3-kinase/AKT pathway and the HPV-mediated pathway represent two mutually exclusive mechanisms which individually affect the tumorigenic contribution of the cyclin D1/CDK4/Rb pathway. However, this possibility could be minor because no association between 3q amplification and HPV infection status

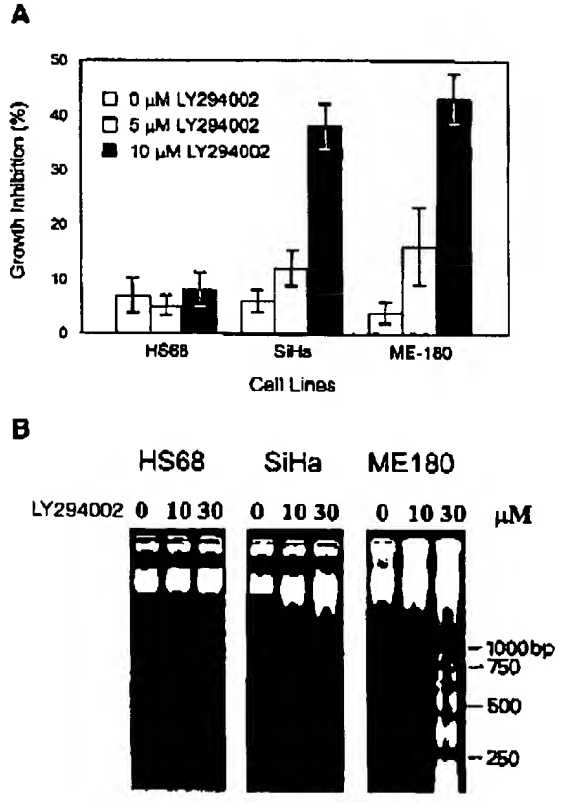

Figure 3 Effect of LY294002, a PI 3-kinase inhibitor, on cell proliferation (a) and apoptosis (b). The cervical cancer cell lines (SiHa and ME180) were found to have amplified *PIK3CA*, increased p110α expression and increased PI 3-kinase activity. The HS68 (normal skin cell line) was known to display only minor p110α expression and PI 3-kinase activity

was found in the present study (data not shown). More intriguing is the fact that both HPV infection and a gain of 3q (associated with abnormal PI 3-kinase/AKT pathway) occur early during cervical tumorigenesis (Ried *et al*., 1999), and, after HPV infection and the existence of dysplastic cervical cells, the appearance of 3q amplification is considered to be the 'obligatory second hit' for the irreversibility of the transformation progress in cervical carcinoma (Ried *et al*., 1999). The reason for the requirement of dual pathways to affect the cyclin D1/CDK4/Rb pathway during cervical tumorigenesis and whether this requirement implies that HPV infection alone is not sufficient to trigger tumor formation, remain to be explored. Future study is needed to explore whether the other major downstream property of cyclin D1/CDK4, e.g. sequestering the CDK inhibitor $p27^{Kip1}$ to trigger cyclin E-initiated Rb-independent S phase entry (Geng *et al*., 1999; Perez-Roger *et al*., 1999; Donnellan and Chetty, 1999), is associated with novel tumorigenic mechanisms caused by amplified *PIK3CA* in cervical cancer development. Not mutually exclusive are the possibilities that, in cervical tumorigenesis, increased *PIK3CA* and elevated PI 3-kinase activity contribute to other AKT-involved growth-promoting pathways or anti-apoptotic signal.

Materials and methods

Tumor specimens from 55 cervical carcinomas were collected. These tumors were exclusively primary site cancers that had not been treated with either chemotherapy or radiation. Almost all (>85%) of the patients had been infected with high-risk HPV (Ku *et al*., 1997; Chu *et al*., 1999). Tissues were flash-frozen in liquid nitrogen, and maintained at −80°C until subsequent analysis. Furthermore, three human cervical cancer cell lines (C-33A, ME-180, and SiHa), one human ovarian cancer cell line (Ovca), one human breast cell line (HBL-100), and one normal human skin line (HS68) were also obtained for the *in vitro* study of *PIK3CA*. The Ovca cell line was known to harbor amplified *PIK3CA* (Shayesteh *et al*., 1999), serving as a positive control, whereas the HBL-100 was known to be negative of *PIK3CA* amplification. These cells were cultured in Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 mcg/mL), and L glutamine (2 mM) in a humidified incubator with 5% CO_2.

The following sequential experiments were used to examine the role of *PIK3CA* in cervical carcinoma: (i) CGH was used to define chromosomal aberration profiles, including 3q26 (where *PIK3CA* is located) amplification, in 18 primary cervical tumor tissues (randomly selected from the 55 cervical carcinomas mentioned above) and all cell lines. Methods were performed as described at http://www.nhgri.nih.gov/DIR/LCG/CGH/technology.html. Analysis of chromosomal aberrations during metaphase was performed using a sensitive monochrome CCD camera and automated image analysis software. The system measures the green(normal)-to-red-(tumor) ratios along the entire length of each chromosome. Average green/red fluorescence intensity ratio profiles were calculated for each chromosome in 8–10 metaphases. DNA gain and amplification in the test DNA were seen as chromosomal regions with an increased fluorescence ratio, while loss and deletion resulted in a reduced ratio. The cut-off lines of increased and reduced ratios were 1.2 and 0.8; (ii) Specific increase of *PIK3CA* copy number in 55 primary tissues and cell lines was measured by competitive PCR (PCR MIMICS) (Siebert and Larrick, 1993). Reactions were carried out using 50 ng of genomic DNA for each sample, and eight twofold dilutions of *PIK3CA* MIMICS (Clontech, Palo Alto, CA, USA). PCR MIMICS were internal standards represented by non-homologous DNA fragments with primer templates that are recognized by a pair of *PIK3CA*-specific primers (5′-TATTTCATGAAACAAATGA-3′ and 5′-TGCTGTAAATTCTAATGCTG-3′) (Volinia *et al*., 1994). The samples were run on a 2% agarose gel after 28 cycles of PCR, and products were stained with ethidium bromide and the gel photographed. The peak areas corresponding to the sample DNA for *PIK3CA* (221 bp) and *PIK3CA* MIMICS (424 bp) were determined using a densitometer. The log of the ratios of the *PIK3CA* target peaks was compared with the peak areas of *PIK3CA* MIMICS, and plotted against the reciprocal of the log of the amount of *PIK3CA* MIMICS added to the PCR reaction. Linear regression analysis of the eight data points was performed. The amount of *PIK3CA* was calculated by extrapolating from the linear regression. A similar procedure was performed for the β-globin gene. The relative copy number of *PIK3CA* gene was reported as the ratio of *PIK3CA* DNA to β-globin DNA. Furthermore, the change of copy number of the other gene located at 3q26.3, i.e. the telomerase RNA gene (*hTR*) (Feng *et al*., 1995), was also measured using the same method; (iii) The increase of gene product of *PIK3CA*, i.e. p110α, in cell lines was detected by Western blot analysis as previously described (Shayesteh *et al*., 1999). The membrane blots containing protein extracted from cells were incubated with specific antibody reagents, including anti-PI 3-kinase p110α or Cdc2 polyclonal antibodies (Santa Cruz, CA, USA). Specific antibody-binding bands were revealed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG. Protein bands of p110α and Cdc2 were detected by enhanced chemiluminescence detection reagent (Amersham). Membrane hybridized with anti-Cdc2 antibody served as an internal control; (iv) The kinase ability to phosphorylate PI *in vitro* was measured for p110α protein immunoprecipitated from cellular lysates of cell lines with known *PIK3CA* copy number as previously described (Shayesteh *et al*., 1999); (v) The biological effects of increased *PIK3CA*, including promoting cell growth or inhibiting apoptosis, were evaluated by treating cell lines with the PI 3-kinase inhibitor, LY294002. If the cells harbored increased *PIK3CA*, the ability of cells to increase proliferation or decrease apoptosis would be affected by such treatment. The inhibition of cell growth by LY294002 was determined by colorimetric quantitation of viable cells as previously described (Shayesteh *et al*., 1999). For apoptosis assay, cellular DNA fragments were isolated, and, after the treatment of LY294002 of different concentrations, adherent and non-adherent cells were pooled and lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM EDTA and 1% NP-40. After centrifugation, the supernatant was collected, and the extraction of pellets was repeated twice. Pooled supernatants were brought into 1% SDS, digested with DNase-free RNase A (final concentration, 0.2 μg/μL) at 50°C for 1 h followed by proteinase K (final concentration 2.5 μg/μL) digestion at 37°C overnight. Then, DNA was isolated by phenol/chloroform extraction and ethanol precipitation, and was dissolved in 10 mM Tris-HCl/1 mM EDTA solution (pH 7.6). The DNA preparations were separated in 2% agarose gel by electrophoresis and stained with ethidium bromide.

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